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Emergence of Distinct Multiarmed Immunoregulatory Antigen-Presenting Cells during Persistent Viral Infection

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SUMMARY

During persistent viral infection, adaptive immune responses are suppressed by immunoregulatory factors, contributing to viral persistence. Although this suppression is mediated by inhibitory factors, the mechanisms by which virus-specific T cells encounter and integrate immunoregulatory signals during persistent infection are unclear. We show that a distinct population of IL-10-expressing immunoregulatory antigen-presenting cells (APCs) is amplified during chronic versus acute lymphocytic choriomeningitis virus (LCMV) infection and suppresses T cell responses. Although acute LCMV infection induces the expansion of immunoregulatory APCs, they subsequently decline. However, during persistent LCMV infection, immunoregulatory APCs are amplified and parallel the viral replication kinetics. Further characterization demonstrates that immunoregulatory APCs are molecularly and metabolically distinct, and exhibit increased expression of T cell-interacting molecules and negative regulatory factors that suppress T cell responses. Thus, immunoregulatory APCs are amplified during viral persistence and deliver inhibitory signals that suppress antiviral T cell immunity and likely contribute to persistent infection.

INTRODUCTION

The immune system resolves the majority of viral infections through a concerted effort of both innate and adaptive mechanisms. These multifaceted responses result in effective elimination of viral pathogens and the establishment of long-lasting protective immunity. However, some viruses, including human immunodeficiency virus (HIV) and hepatitis C virus (HCV) in humans and lymphocytic choriomeningitis virus (LCMV) in mice, are capable of enduring the initial immune onslaught and establishing persistent infections (Klenerman and Hill, 2005; Wilson and Brooks, 2010). The prolonged and elevated viral titers associated with these types of infection progressively alter T cell responses in a phenomenon known as exhaustion (Fahey and Brooks, 2010). While immune exhaustion is counterproductive to viral clearance, it is likely necessary to prevent harmful bystander immunopathology that is associated with prolonged T cell responses in the face of unresolved high-level virus replication (Barber et al., 2006; Yi et al., 2009). Exhausted T cell responses have a unique developmental program characterized by decreased proliferation and the diminished ability to produce antiviral and immunostimulatory cytokines that are associated with acute viral infections (Fahey et al., 2011; Wherry et al., 2003; Wherry et al., 2007). Importantly, some degree of lingering T cell activity is actively maintained in persistent infection, and while these residual responses differ in cytokine production and magnitude from what is considered to be fully productive antiviral T cell responses, they are critical for the long-term control of viral replication (Agnellini et al., 2007; Elsaesser et al., 2009; Fahey et al., 2011; Frohlich et al., 2009; Yi et al., 2009). Thus, elucidating the control mechanisms that modulate T cell responses will be important toward understanding how these pathogens subvert the immune response to persist.

At the onset of an infection, T cells are primed by specialized antigen-presenting cells (APCs) called dendritic cells (DCs) (Banchereau and Steinman, 1998). During the initial priming, multiple interactions, including signals from surface-bound and soluble costimulatory and/or inhibitory molecules, function in concert to stimulate and fine-tune T cell responses. However, since these initial interactions cannot forecast the long-term immune requirements needed to fight a particular infection, cellular responses are pliant to local signals, and T cell functions are continually modulated in response to the needs of the current antigenic environment (Brooks et al., 2006a). As a result, multiple APCs (including macrophages and B cells) and infected cell populations (Mueller et al., 2007) that are not potent inducers of T cell activation likely have important roles in the modulation of the immune response as infection progresses. This is particularly relevant during persistent infections both early, as the initially productive T cell responses are suppressed, as well as during viral persistence to modulate T cell activity and protect

from immunopathology while continuing to battle the infection (Barber et al., 2006; Brooks et al., 2006b).

The host-derived immunoregulatory cytokine IL-10 is crucial in driving T cell exhaustion and viral persistence following LCMV infection (Brooks et al., 2006b; Ejrnaes et al., 2006). Early disruption of IL-10-mediated suppression prevents the loss of T cell activity in response to an otherwise persistent LCMV infection leading to rapid virus clearance (Brooks et al., 2006b; Ejrnaes et al., 2006). Similarly, elevated IL-10 levels correlate with HIV replication in humans, and recently a link between IL-10 expression and transition into persistent HCV infection was identified (Brockman et al., 2009; Flynn et al., 2011). Ex vivo IL-10 blockade enhanced anti-HIV and anti-HCV T cell activity (Clerici et al., 1994; Landay et al., 1996; Rigopoulou et al., 2005), further indicating the important and conserved suppressive role of IL-10 during many persistent virus infections.

In addition to IL-10, multiple immunoregulatory pathways actively, simultaneously, and increasingly suppress T cell activity during viral persistence, including PDL1/PD1, Lag3, Tim3, CTLA4, indoleamine 2,3 dioxygenase (IDO), and TGF-β (Wilson and Brooks, 2011). Consistent with graded levels of T cell exhaustion, wherein increasing amounts of inhibitory signals in combination achieve a threshold necessary for functional T cell suppression, the concurrent blockade of multiple suppressive signals additively enhances antiviral T cell activity (Blackburn et al., 2009; Brooks et al., 2008; Jin et al., 2010). Yet, it is unclear how the concurrent inhibitory signals needed to achieve the suppressive threshold are delivered to a specific T cell in the context of an overwhelming virus infection and inflammatory environment. Herein we identify specific populations of immunoregulatory APCs (which for simplicity in nomenclature will be called iAPCs) that simultaneously produce multiple T cell-interacting and immune-inhibitory molecules to suppress antiviral T cell activity. Consistent with the expression of suppressive factors, iAPCs are invoked by virus replication but rapidly wane in the stimulatory environment of an acute infection. Conversely, iAPCs are potentiated and highly amplified during persistent infection to facilitate dampening of T cell responses and maintenance of the suppressive environment. Thus, the amplification and clustering of multiple immunoregulatory factors on a single iAPC is a mechanism to simultaneously deliver potent inhibitory signals and in a single interaction tip the balance toward exhausted T cell responses and viral persistence.

RESULTS

Enhanced IL-10 Production during Viral Persistence Is Primarily of Hematopoietic Origin

To elucidate the dynamics of IL-10 expression in viral infection, we utilized the LCMV model system. Infection with the Armstrong (Arm) variant of LCMV induces robust CD4 and CD8 T cell responses that clear the virus within the first 2 weeks after infection. Alternatively, the Clone 13 (Cl 13) variant of LCMV replicates to substantially higher titers and, although initially inducing productive T cell responses, rapidly elicits the expression of multiple host immunoregulatory factors that suppress T cell activity to generate a persistent infection (Ahmed et al., 1984; Wilson and Brooks, 2011). A unique aspect of the LCMV system is the ability to directly compare an acute and persistent infection with the same virus, allowing for the differentiation of factors that are increased and important in persistent infection from those that are the result of a common response to viral infection.

To specifically characterize mechanisms of IL-10-mediated immunosuppression, we utilized the Vert-X IL-10 reporter mouse which expresses green fluorescent protein (GFP) linked by an internal ribosome sequence (IRES) to the IL-10 locus (Madan et al., 2009). Vert-X mice allow for direct identification of IL-10-expressing cells without in vitro manipulation or cell fixation. Regulation of IL-10 expression is not perturbed in these mice, and the course of LCMV infection in this strain is analogous to WT mice (data not shown). Importantly, IL-10 RNA and protein expression was dramatically elevated in the GFP+ cells (see Figure S1 online). Additionally, analyses described herein were confirmed using a second IL-10 reporter (10BiT) mouse (Maynard et al., 2007). The 10BiT mouse contains a bacterial artificial chromosome (BAC) with a Thy1.1 reporter cassette inserted in-frame in the first exon of the IL-10 locus along with a stop codon, thus enabling identification of IL-10-expressing cells without any manipulation of the two functioning (i.e., no IRES) endogenous IL-10 genes. All data were reproducible and analogous in the 10BiT mice (data not shown).

IL-10 protein was detectible in the plasma from both Arm- and Cl13-infected mice at early time points; however, upon the resolution of acute infection, IL-10 levels wane, whereas they remain elevated throughout the course of a persistent infection (Figure 1A). While many cell types can produce and respond to IL-10 in varying situations, the relevant sources in persistent viral infection are still controversial (Wilson and Brooks, 2011). To begin to define the relevant sources of IL-10 in persistent infection, we generated reciprocal mixed bone marrow chimeras of B6- and IL-10-deficient mice. Following persistent Cl13 infection, plasma IL-10 levels were elevated only when the hematopoietic compartment was IL-10 sufficient (Figure 1B). On the other hand, IL-10 levels were low to undetectable when the hematopoietic compartment was derived from IL-10-deficient mice, despite the rest of the mouse being IL-10 sufficient, indicating that the induction of IL-10 during viral persistence is predominantly from hematopoietic-derived cells. Production of IL-10 from hematopoietically derived cells was elevated in multiple organs throughout persistent compared to acute infection (Figures 1C and 1D). GFP+ gates were based on control nontransgenic B6 mice (Figure S1). Interestingly, the absolute number of GFP-expressing cells was elevated in persistent infection, despite the overall decrease in splenic cellularity relative to acute infection (Arm, $3.9 \times 10^7 \pm 1.2 \times 10^7$, versus Cl13, $2.5 \times 10^7 \pm 9 \times 10^6$; $p = 0.02$). In other tissues, the percentage of IL-10-expressing cells was elevated during persistent compared to acute infection, but the lower overall levels of cellularity during persistent infection led to equal overall numbers of iAPC (Figure 1D). Levels of IL-10-expressing cells rose and peaked in the spleen around day 9 after LCMV-Cl 13 infection (Figures 1C and 1D) in conjunction with the transition from functional to exhausted T cell responses (Brooks et al., 2006a). IL-10-expressing cells in the lymph nodes and in multiple nonlymphoid organs were initially lower, but then maintained or increased through persistent infection, whereas they remained low in acute infection (Figures 1C and 1D).

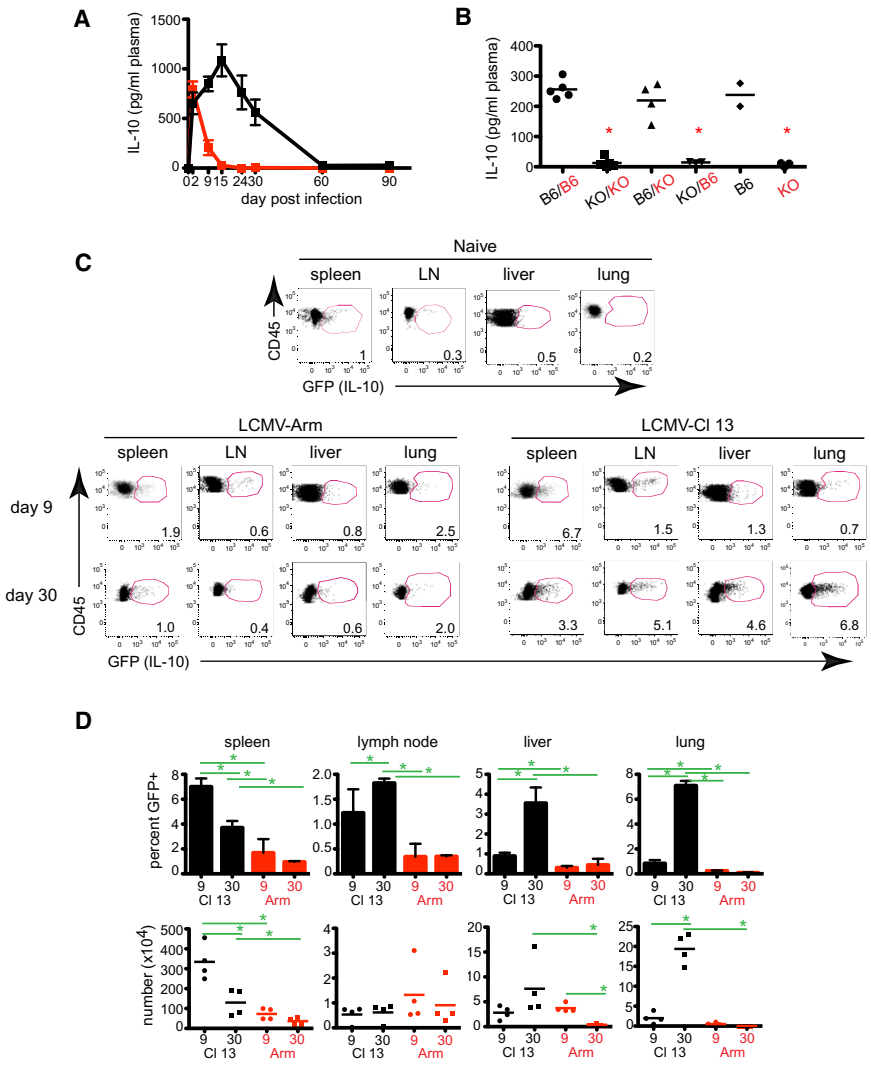

Figure 1. IL-10 Production during Viral Persistence Is Primarily of Hematopoietic Origin

(A) Kinetics of IL-10 protein concentration in the plasma at the indicated days after LCMV Arm (red) or LCMV CL13 (black) infection of Vert-X mice.

(B) Bone marrow chimeric mice were generated by irradiating recipient mice (red) and reconstituting with donor bone marrow (black). Nonirradiated control C57BL/6 (B6) and IL-10-deficient (KO) mice were included for comparison. Plasma IL-10 levels 15 days after LCMV Cl13 infection.

(C) Time course of IL-10 (GFP) expression in multiple organs during acute (LCMV-Arm) and persistent (LCMV-Cl13) infection.

(D) Bar graphs represent the percentage (upper graphs) and number (lower graphs) of GFP-expressing cells at days 9 and 30 following acute or persistent infection. Symbols represent individual mice and are representative of three to five mice per group and two or more independent experiments. Error bars represent ± standard deviation (SD) *p = < 0.05. See also Figure S1.

IL-10-Producing APCs Are Amplified in Persistent Infection and Express Antigen Presentation and Costimulation Machinery

To define the mechanisms of how IL-10 suppresses the immune response to facilitate viral persistence, specific cell subsets that exhibit enhanced IL-10 expression in persistent as compared to acute infection were quantified. At the peak of IL-10 expression in the spleen, multiple immune subsets produced IL-10, including DCs, macrophages, B cells, and $CD4^+$ and $CD8^+$ T cells (Figure 2A). GFP-positive gates were again set using non-transgenic B6 control mice (Figure S2). Interestingly, both numerically and as a percentage, macrophages comprised the highest amount of GFP+ (IL-10-producing) cells. Of note, the level of IL-10-producing CD4 and CD8 T cells was similar at the initial stages of acute and persistent infections, and the vast majority of virus-specific T cells (i.e., tetramer positive) did not produce IL-10. GFP expression was not observed in NK cells or neutrophils (data not shown). While most of the cell subsets contained IL-10-producing populations at day 9 postinfection, the percent and number of IL-10-producing APCs (DCs, macrophages, and B cells) were substantially higher in persistent infection and continued to remain elevated throughout viral persistence (Figure 2B). Thus, although multiple cell subsets produce IL-10 in response to viral infection, there was a distinct difference in the regulation of APC populations during acute and persistent infection.

DCs are required to initially activate naive antiviral CD4+ and CD8+ T cell responses in vivo (Jung et al., 2002; Probst and van den Broek, 2005). However, depletion of DCs after priming (but before loss of T cell function) following LCMV-Cl 13 infection did not prevent T cell exhaustion (Figure S2). We previously demonstrated that T cells retain function when removed from LCMV-Cl 13 infection at this same time (Brooks et al., 2006a), indicating that as infection progresses, alternative APCs in addition to DCs, such as macrophage and B cells, play important regulatory roles to modulate previously activated T cell responses and adjust the immune response to the needs of the evolving antigenic environment. Therefore, we next sought to investigate the regulatory properties of IL-10-producing APCs during persistent infection. The majority of IL-10-producing DCs were $CD11c^{bright}$, $CD11b^{hi}$ CD8 α^{lo}, $B220^{neg}$, so we subsequently compared IL-10+ versus IL-10- DC within this subset; however, a smaller yet evident population of $CD11c^{bright}$, $CD11b^{lo}$ CD8 α^{hi}, $B220^{neg}$ DC also produced IL-10 (Figure S2). Of note, similar results as described below were observed when IL-10+ versus IL-10- CD8+ DCs or total DCs were compared (data not shown). IL-10-expressing B cells are not limited to a single B cell subset and are observed in fractions of plasma cells, memory cells, marginal zone cells, and germinal center compartments (data not shown). Compared to their non-IL-10-producing counterparts, IL-10-producing DCs, macrophages, and B cells expressed equal if not enhanced

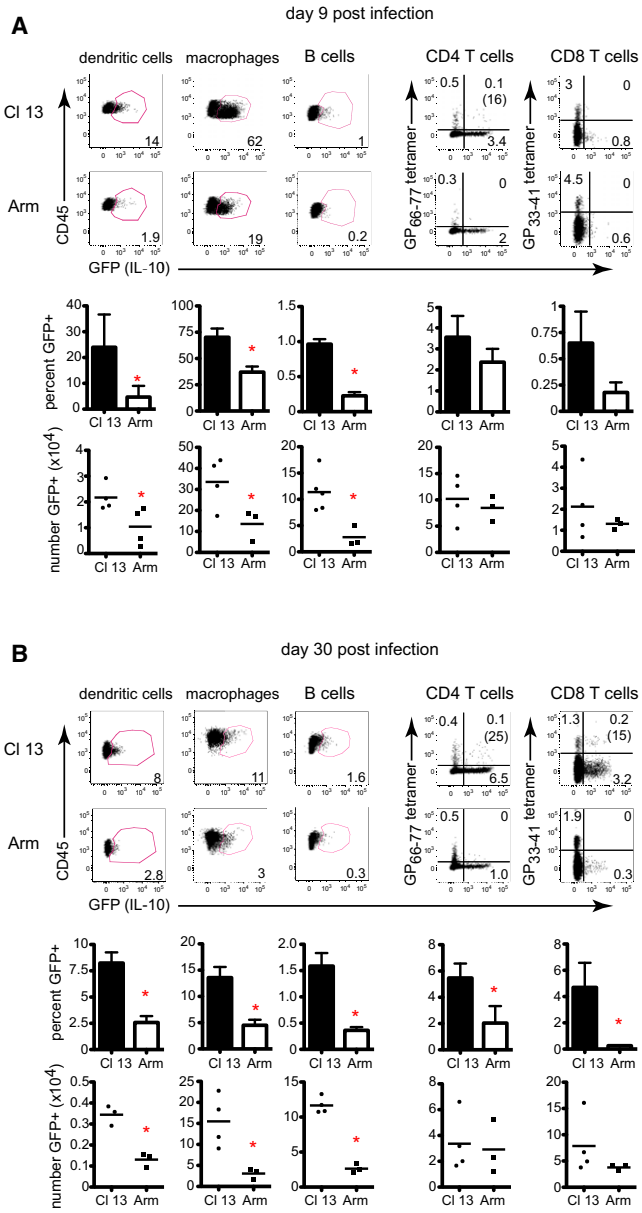

Figure 2. Multiple APC Subsets Produce IL-10 in Persistent Infection

Flow plots depict GFP (IL-10) expression in Vert-X mice on (A) day 9 or (B) day 30 following LCMV-Cl 13 (top, black bars) or LCMV-Arm (bottom, white bars) infection. Numbers indicate the percentage of GFP+ cells in each subset, and graphs indicate the average percentage or number ±SD of GFP+ cells. Gating strategy for each population is such that GFP signal is less than 1% in wild-type C57BL/6 control mice (see Figure S1). *$p < 0.05$. Data are representative of four mice per group and four independent experiments. See also Figure S2.

levels of MHC class I and II proteins as well as the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) (Figures 3A–3C). The small numbers of IL-10-expressing APCs present in acute infection (Figure 2) were of similar phenotype and DC subset distribution (Figures S2 and S3). Taken together, these results indicate that IL-10-producing APCs are amplified in persistent infection and represent mature populations capable of interacting with and potentially modulating T cell responses.

IL-10-Producing APCs Simultaneously Express Multiple Immunosuppressive Factors

Numerous regulatory mechanisms in addition to IL-10 have evolved to restrain immune activity during persistent infection (Wilson and Brooks, 2011). Exemplary of these are PD-1/PDL1 interactions and the critical role these host-derived proteins play in sustaining T cell suppression in persistent viral infections (Barber et al., 2006). Interestingly, both PDL1 and PDL2 were significantly upregulated on all IL-10-expressing APC populations compared to their non-IL-10-producing counterparts (Figure 4A and Figure S4) and subsequently maintained throughout the course of persistent infection (Figure 4A), suggesting that multiple inhibitory factors may be centralized onto single iAPC subsets.

To further explore the functions of iAPC populations, we assessed expression of other immunosuppressive molecules implicated in suppressing immune responses during persistent infection. IDO dampens T cell responses via modulation of tryptophan catabolism (Mellor and Munn, 2004). *IDO* expression was substantially elevated in IL-10-producing DCs, macrophages, and B cells relative to their non-IL-10-producing counterparts (Figure 4B). TGF-β is another immunoregulatory cytokine recently indicated to limit antiviral T cell responses and facilitate LCMV persistence (Tinoco et al., 2009). However, unlike other immunosuppressive factors, iAPCs were not enriched for *TGF-β* transcripts (Figure 4B), indicating that iAPCs do not universally upregulate all immunosuppressive genes important during viral persistence. APC production of the potent proinflammatory cytokine IL-12 is a key component in the induction of IFNγ-producing T cell responses (O'Garra, 1998). RT-PCR analysis in iAPC subsets indeed revealed a dramatic reduction in *IL-12p35* levels when iAPCs were compared to their non-IL-10-expressing counterparts (Figure 4B). Thus, in addition to increased expression of negative regulatory factors, iAPCs also produced decreased levels of positive stimulatory factors.

The increased expression of T cell-interacting proteins and immunosuppressive factors in conjunction with lower levels of stimulatory cytokines suggested that iAPCs in particular would diminish instead of stimulate T cell responses. In accordance with their inability to prime naive T cells, neither B cells nor macrophages derived directly from persistent infection (regardless of IL-10 expression) were able to stimulate naive T cells ex vivo (data not shown). However, consistent with their immunosuppressive phenotype, iDC suppressed virus-specific T cell responses ex vivo compared to their non-IL-10-producing counterparts (Figure 4C and Figure S4). T cell-stimulatory capacity was enhanced in iDC, but not non-iDC, when IL-10, PDL1, or IDO signaling was blocked (Figure 4C and Figure S4). Thus, iAPCs express concentrated levels of inhibitory molecules with the ability to independently suppress virus-specific T cell responses.

Immunoregulatory APC Maintenance and Amplification Correlates with Virus Replication Kinetics but Not Direct Infection

Multiple immunosuppressive molecules are similarly upregulated early in acute and persistent virus infection; however, expression of these factors diminishes in an acute infection,

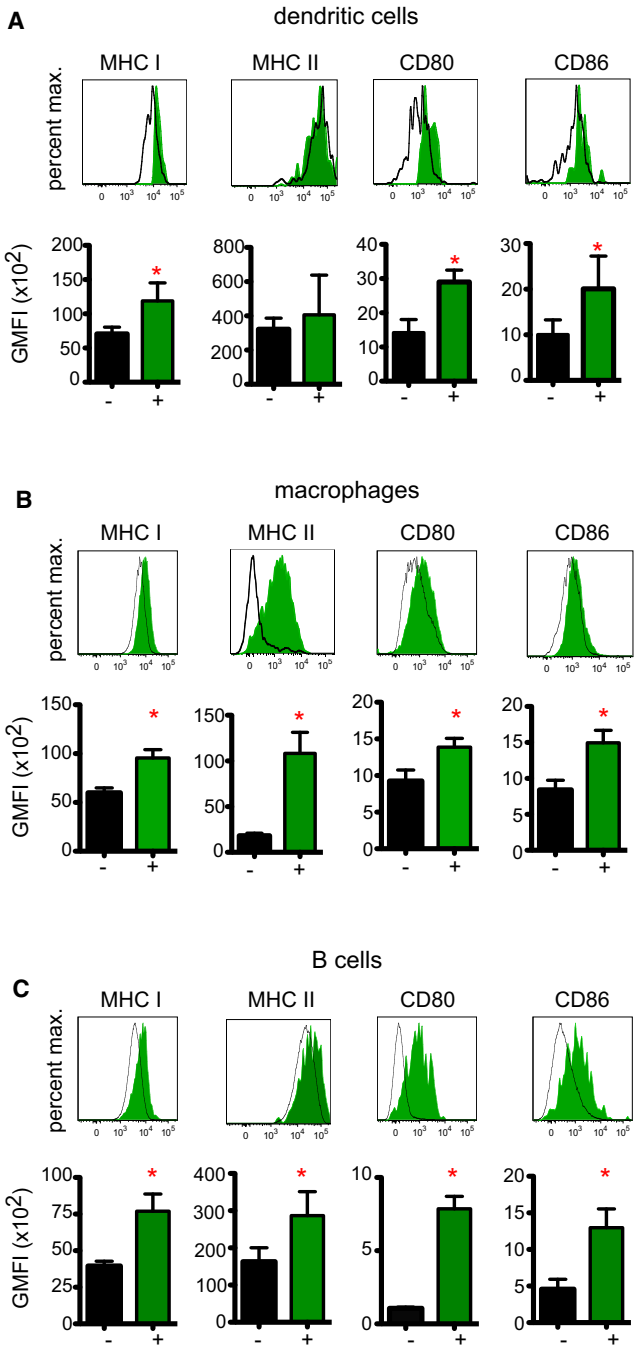

Figure 3. APCs Express Antigen Presentation and Costimulation Machinery

Flow cytometric analysis comparing IL-10 producing (green) and non-IL-10 producing (black) in (A) DCs, (B) macrophages, and (C) B cells from the spleens of Vert-X mice 9 days after LCMV Cl13 infection. Representative histogram overlays between GFP+ (green) and GFP− (black) cells are shown. The corresponding bar graphs represent the geometric mean intensity (GMFI) ±SD of four to five mice per group from one of three independent experiments. *$p < 0.05$. See also Figure S3.

allowing T cells to maintain functionality (Barber et al., 2006; Brooks et al., 2006b). Conversely, at the same time that negative regulatory factors wane during acute infection, their expression is sustained and amplified in persistent infection, promoting T cell exhaustion and viral persistence (Figure 1A and Brooks et al., 2006b; Ejrnaes et al., 2006). Yet, the reasons for the simultaneous sustained and increased expression of these multiple factors are unclear. Interestingly, the expression kinetics of iAPCs in acute and persistent infection parallels this course, being initially equal when virus titers are similar (day 5) and then waning to very low levels with decreased virus replication in acute infection and expanding in conjunction with the increasing virus replication in what will become a persistent infection (Figure 5A). Inversely, levels of conventional (non-IL-10-producing APCs) dramatically increased during acute infection, whereas they remained low in response to prolonged virus replication in persistent infection (Figure 5A). As the persistent infection ensues, contraction of iAPCs mirrors the decrease in virus titers (Figure 5A). Thus, iAPCs are invoked in response to elevating virus expression, leading to sustained and heightened expression of multiple immunoregulatory factors associated with immune suppression during viral persistence.

Because of the tight correlation between viral load and iAPC numbers, we sought to determine whether direct infection of APCs was responsible in inducing the iAPC phenotype. While there was a slight increase in the percentage of iAPCs that were infected relative to their nonproducing counterparts, the vast majority of iAPCs were not productively infected (Figure 5B and data not shown), indicating that direct infection is not driving the iAPC phenotype.

Immunoregulatory APCs Are Metabolically, Transcriptionally, and Phenotypically Distinct

Recent studies have implicated an APC's underlying metabolic program in regulating fate and function. Oxidative metabolism appears to be required for the acquisition of an anti-inflammatory or immunoregulatory phenotype (Vats et al., 2006). Conversely, TLR activation of canonical proinflammatory programs in DCs requires a metabolic switch to aerobic glycolysis (Krawczyk et al., 2010). Interestingly, IL-10 signals inhibited the acquisition of TLR-induced glycolytic programs in APC (Krawczyk et al., 2010). To determine if this metabolic paradigm was operative in the context of a persistent viral infection, we quantified the mitochondrial membrane potential of IL-10-producing and IL-10-negative macrophages, DCs, and B cells on day 9 after LCMV-Cl 13 infection. Strikingly, GFP+ macrophages, DCs, and B cells exhibited increased MitoTracker Red staining relative to their GFP− counterparts ex vivo, indicating higher mitochondrial membrane potential (Figure 6A).

The increased mitochondrial potential in IL-10-producing APCs could be indicative of a heightened oxidative metabolic state or the result of increased mitochondrial mass. To distinguish between these two possibilities, we quantified expression of the mitochondrially encoded gene *cytochrome c oxidase II* (*Mt-co2*) relative to the nuclear housekeeping gene nuclear receptor-interacting protein 1 (*Rip140*). The ratio of *Mt-co2* to *Rip140* was not statistically different between IL-10-producing and IL-10-negative DCs and macrophages (Figure 6B), suggesting an equivalent mitochondrial mass in both populations of APCs. The metabolic state of IL-10-producing B cells is less clear, as *Mt-co2* levels are significantly elevated in these

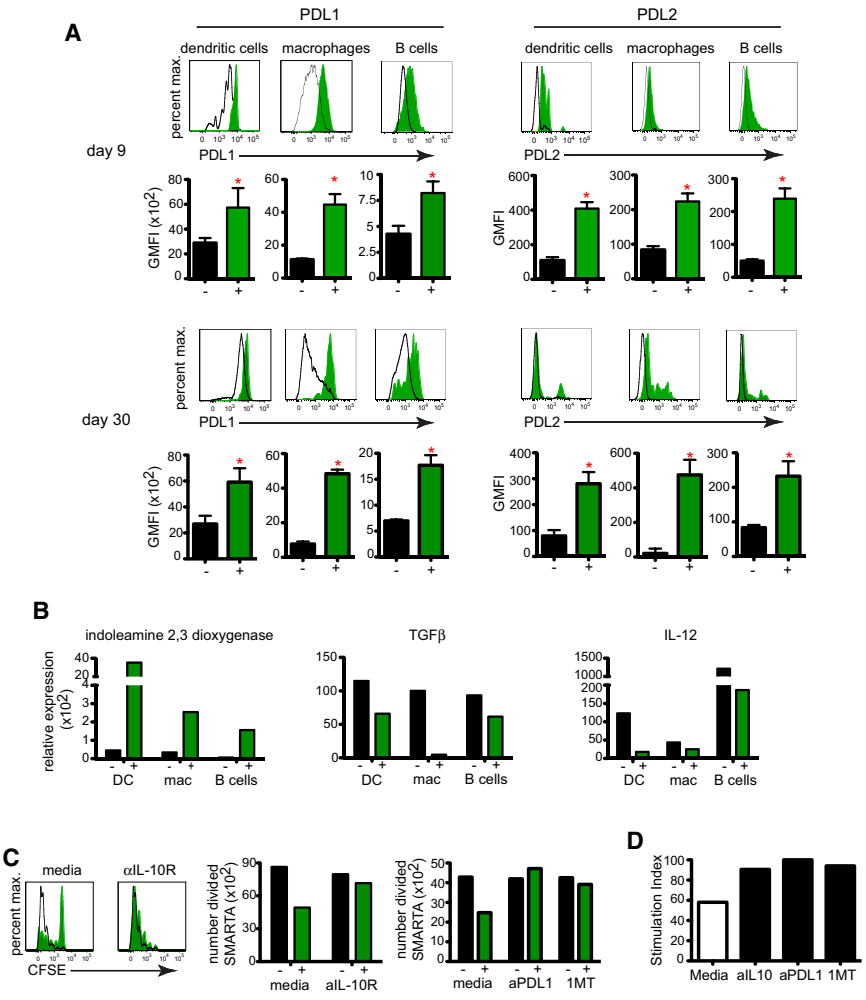

Figure 4. iAPCs Simultaneously Express Multiple Immunoregulatory Molecules and Have Reduced Stimulatory Capacity

(A) Flow cytometric analysis of GFP+ (green) and GFP− (black) splenic DCs (CD11b+), macrophages, and B cells from Vert-X mice on days 9 and 30 after LCMV Cl13 infection. Representative histogram overlays of PDL1 (left) and PDL2 (right) of cells. Bar graphs represent the GMFI ±SD of PDL1 and PDL2 expression by the indicated APC subset; *$p < 0.05$.

(B) mRNA expression of *indoleamine 2,3 dioxygenase* (right), *TGF-β* (middle), and *IL-12* (left) relative to *HPRT* in the indicated IL-10+ (GFP+) or IL-10− (GFP−) APC subset on day 9 after LCMV Cl13 infection. Each group is a pool of cells from six to eight mice and is representative of two or more independent experiments.

(C) GFP+ (CD11b+) DCs (green histogram) or GFP- (CD11b+) DCs (black histogram) were sorted from the spleens of day 9 LCMV Cl13-infected Vert-X mice and cultured for 3 days with naive CFSE-labeled TCR transgenic, LCMV-specific CD4+ (SMARTA) T cells in either media alone or with the addition of anti-IL-10R blocking antibody. Bar graphs represent the number of SMARTA cells that divided in cultures containing media alone or anti-IL-10R, anti-PDL1, or the IDO inhibitor 1-MT. The separate bar graphs represent independent experiments. Each condition is representative of at least two independent experiments.

(D) Stimulation index indicates the total number of SMARTA T cells that proliferated in the cultures with GFP+ (CD11b+) DCs divided by the number of CD4+ SMARTA T cells that proliferated in the GFP− culture in the presence of media alone or of anti-IL-10R, anti-PDL1, or 1MT (a chemical inhibitor of IDO). Each group is a pool of cells from six to eight mice and is representative of two or more independent experiments. *$p < 0.05$. See also Figure S4.

populations—indicating an increase in mitochondrial number as well as potential. We further examined the expression levels of key glycolytic genes. IL-10-producing macrophages expressed markedly lower levels of the glycolysis genes *hexokinase 2* (*HK2*), *lactate dehydrogenase A* (*Ldha*), and *pyruvate kinase* (*Pkm2*) (Figure 6C), indicative of an oxidative/suppressive metabolic program. Interestingly, GFP+ and GFP− DC populations expressed similar levels of the glycolysis genes, suggesting a complex metabolic regulation. Hence, iAPCs are metabolically distinct subsets of cells that simultaneously express multiple immunoregulatory factors with heightened suppressive activity.

The metabolic, phenotypic, and functional attributes of the immunoregulatory macrophages that arise during infection are consistent with those of M2/alternatively activated macrophages (Lawrence and Natoli, 2011). The IL-10-expressing macrophages induced during persistent viral infection express low levels of GR1 (correlating with M2 development; Lin et al., 2009) and are highly enriched for the M2-associated transcripts Arg1 and CD206 (Figure 6D). iAPC populations also exhibit a distinct molecular profile. Interferon-regulatory factors (IRFs) comprise a family of signaling molecules that play important roles in the regulation of M2 macrophage polarization and DC ontogeny (Savitsky et al., 2010; Satoh et al., 2010). Expression of *IRF4* is significantly elevated in IL-10-producing macrophages and DCs, whereas expression of the related interferon-regulatory factor *IRF3* is similar compared to IL-10 nonproducers (Figure 6E). B-lymphocyte-induced maturation protein 1 (Blimp-1) is a transcriptional repressor in T and B cell development (Xin et al., 2011), promotes IL-10 production, and is linked to maintenance of tolerogenic DC populations (Chan et al., 2009; Kim et al., 2011; Martins et al., 2006). Interestingly, *Blimp1* expression was highly elevated in IL-10-expressing, compared to nonexpressing, DCs and macrophages (Figure 6E). In accordance with the antagonism between Bcl6 and Blimp1, *Bcl6* expression was decreased in iAPCs (Figure 6E). Thus, persistent infection promotes the amplification of iAPC populations with distinct molecular and metabolic profiles that simultaneously express multiple immunosuppressive and T cell-modulating factors (IL-10, PDL1, PDL2, B7-1, B7-2, and IDO) capable of negatively regulating antiviral T cell activity.

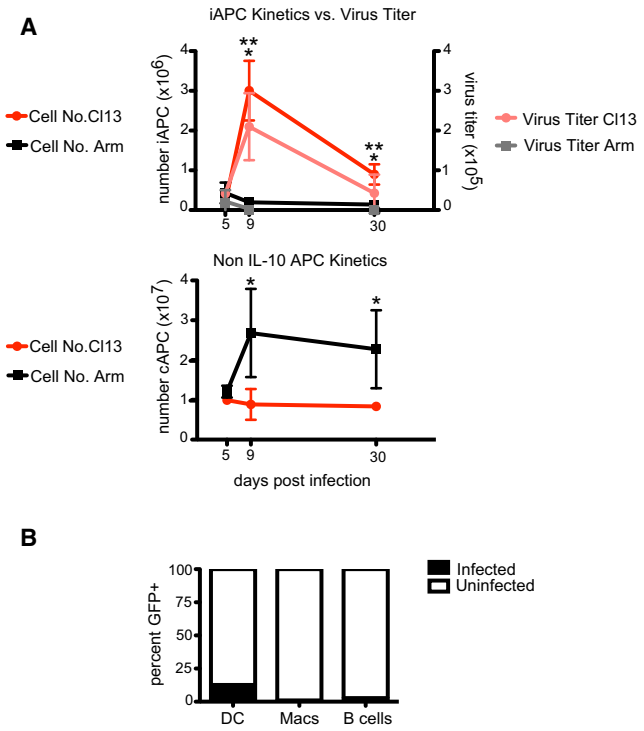

Figure 5. iAPCS Tightly Correlate with Levels of Virus Replication but Are Not Directly Infected

(A) Quantification of total IL-10-expressing APCs (DCs, macrophages, and B cells) compared to virus replication kinetics in acute LCMV-Arm (black and gray lines) and LCMV-Cl 13 (red and pink lines) infection. Data are representative of four to five mice per group and two or more independent experiments. *$p < 0.05$ comparing number of iAPCs in LCMV-Arm versus LCMV-Cl13 infection. **$p < 0.05$ comparing virus titers in LCMV-Arm versus LCMV-Cl13 infection. Error bars represent ±SD.

(B) IL-10-expressing DCs, macrophages, and B cells were FACSorted, and the percent of each population infected was quantified. Data are representative of pools of six mice per group and two independent experiments.

DISCUSSION

Activation of effective CD4 and CD8+ T cell responses in the face of persistent viral infection is a major therapeutic goal. However, the multiple immunoregulatory pathways that are elevated and sustained during persistent infection, resulting in T cell suppression, must be overcome while not engendering widespread immunopathology. In this study we demonstrate that many immune cell types simultaneously produce IL-10, potentially all playing important and distinct roles in suppressing the host immune response. Interestingly, within each APC population we identified a distinct subset that coexpressed many of the dominant factors implicated in suppressing T cell responses in viral persistence. These iAPC subsets have similarities to other previously described populations (i.e., M2 macrophages) that can arise during other states of chronic inflammation. Importantly, these specialized iAPCs are dramatically amplified during persistent infection and peak during the transition into T cell dysfunction.

Our results demonstrate that invocation of iAPCs is a fundamental and previously unrecognized response to viral replication. Paradoxically, the same inhibitory factors that are responsible for inducing viral persistence are also present early in acute infection, but then rapidly disappear to facilitate productive T cell responses (Figure 1A, Figure 5A, and Barber et al., 2006; Brooks et al., 2006b). On the other hand, these same factors are sustained and amplified in what will become a persistent infection, promoting immune suppression and viral persistence. Thus, an inflection point exists when the immune response determines whether it is winning or losing the battle with viral replication and then adjusts itself accordingly. Interestingly, dynamic regulation of iAPC at the inflection point between virus clearance and persistence clarifies the enigmatic expression kinetics of multiple inhibitory factors. Based on the important role of these multiple inhibitory factors in inducing and maintaining T cell/immune exhaustion, the coexpression of these molecules by single cells that can themselves be rapidly regulated is a mechanism whereby the immune response can be quickly adjusted to appropriately modulate aggressive immunity when the battle is being won, with suppressive responses that prevent excessive immunopathology when the battle is lost. However, the immune response is not binary (i.e., vigorous effector responses or complete exhaustion), and by varying the levels of iAPCs, it may be possible to achieve the spectrum of T cell (and overall immune) quality/quantity required to fight a given pathogen. Importantly, targeting of these cells may represent an effective therapeutic approach to delete immunosuppressive cells while maintaining stimulatory APCs to sustain ongoing immune responses.

iAPCs exhibit a separate molecular, metabolic, phenotypic, and functional profile compared to their conventional APC counterparts residing in the same environment, indicating that they are a distinct group of cells that naturally arise during viral infection and are specifically and dramatically amplified in response to persistent infection. Further, despite belonging to different APC cell types (i.e., DCs, macrophages, B cells), the similar molecular profile of iAPCs suggests that they may share common triggers and differentiation programs in response to ongoing virus replication. The direct correlation of iAPC numbers with virus replication kinetics suggests that iAPCs may not be sustained individually long-term as much as continually stimulated. Based on the known plasticity of macrophages (and potentially DCs, either through functional modification or rapid turnover), it is possible that APCs are continually recruited into the immunoregulatory subset to appropriately modulate the current needs of the immune environment. However, the fact that blockade of IL-10, PDL1, and other negative regulatory factors successfully enhances effector T cell responses during persistent infection indicates that these iAPC populations continue to have an important immune-modulatory role throughout the course of such infections.

The concurrent blockade of multiple suppressive signals additively enhances antiviral T cell activity (Blackburn et al., 2009; Brooks et al., 2008; Jin et al., 2010), suggesting the existence of a threshold level of duration and/or strength of signals that must be reached by a particular cell to initiate the exhaustion program. It is important to note that inhibitory factors may possess different levels of activity during persistent infection. In vivo inhibition of IL-10 or PDL1 can prevent or decrease persistent virus replication, while blockade of other immunoregulatory molecules in vivo such as CTLA4 have less obvious impact (Barber et al., 2006; Kaufmann et al., 2007), potentially

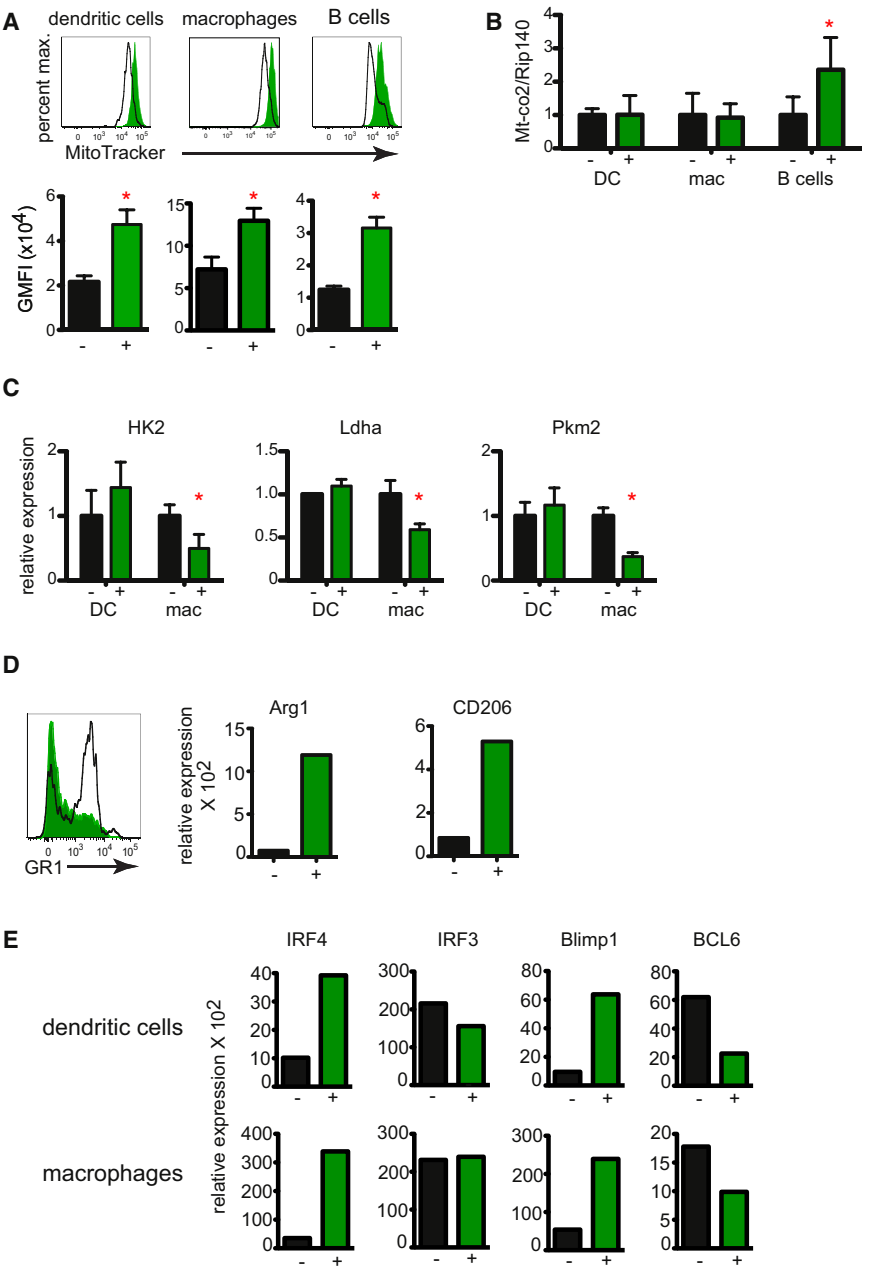

Figure 6. IL-10-Expressing APCs Have Altered Metabolic Programs

(A) Mitochondrial membrane potential assessed by MitoTracker FM RED staining of IL-10+ or IL-10− macrophages, DCs, and B cells on day 9 after LCMV Cl13 infection. Black histograms/bars are IL-10−, and green histograms/bars are IL-10+ subsets. Data are representative of four to five mice per group and three separate experiments.

(B) Ratio of mitochondrial Mt-co2 to nuclear Rip140 gene expression from purified IL-10+ or IL-10− macrophages, DCs, and B cells on day 9 after LCMV Cl13 infection.

(C) Expression of the glycolysis genes *pyruvate kinase* (*Pkm2*), *lactate dehydrogenase A* (*Ldha*), and *hexokinase 2* (*HK2*) on day 9 after LCMV Cl13 infection.

(D) The histogram illustrates GR1 expression, and the bar graphs represent the expression of M2/alternatively active genes Arginase 1 and CD206 by IL-10+ (green) and IL-10− (black) macrophages. Error bars represent ±SD.

(E) Expression of the signaling/transcription factors *IRF4*, *IRF3*, *Blimp1*, and *BCL6* by the indicated APC populations. Each group is a pool of cells from six to eight mice and is representative of five independent experiments. *$p < 0.05$.

serving to fine-tune the ongoing response. Thus, immunosuppression is a multifactorial process in which many factors contribute to varying degrees (likely through targeting different cells and distinct cellular functions) to cumulatively restrain immune activity (Blackburn et al., 2009). By this mechanism, the clustering of multiple immunoregulatory factors on iAPCs provides a method for rapid delivery of numerous potent inhibitory signals directly to individual T cells.

Antibody blockade of IL-10, PDL1, or other factors during persistent infections enhances T cell activity (Barber et al., 2006; Brooks et al., 2006b; Ejrnaes et al., 2006; Fahey and Brooks, 2010). This underscores the fact that maintenance of exhaustion as an active process and that dynamic alterations in postpriming immune environment are critical modulators of T cell activity. In addition to IL-10-expressing DCs, we also identified the emergence of B cells and a large percentage of macrophages that express IL-10 in concert with many other inhibitory factors that suppress antiviral immunity during persistent infection. When T cells are removed after priming, but prior to loss of function, from what will become a persistent infection, they retain effector activity and differentiate into memory cells (Brooks et al., 2006a). Paralleling this, our finding that DC deletion after priming, but before loss of T cell activity, does not prevent T cell exhaustion indicates that multiple iAPC populations modulate ongoing T cell responses to potentiate T cell exhaustion. Consequently, while not capable of priming naive T cells, IL-10-expressing macrophages and B cells likely play critical roles as infection progresses. In this manner, IL-10-expressing macrophages and B cells express high levels of MHC molecules, fostering their ability to interact with T cells. How each of these iAPCs targets distinct aspects of the immune response to promote persistent infection is undoubtedly complex, but will ultimately be of tremendous importance for the development of targeted therapies to overcome the mechanisms viruses utilize to subvert immune clearance.

Studies examining the metabolic programs adopted by APCs in the context of infection and immunity are limited. Elegant studies by Chawla and colleagues demonstrated that expression

of an oxidative metabolic program was an absolute requirement for the polarization of M2 or alternatively activated macrophages in chronic inflammatory models (Vats et al., 2006). In contrast, activation of DCs by proinflammatory signals induces a metabolic switch from oxidative to glycolytic metabolism which, if pharmacologically inhibited, alters T cell-stimulatory capacity (Krawczyk et al., 2010). In our study, we observed that IL-10-producing macrophages have significantly higher membrane potential (with no appreciable change in mitochondrial mass and decreased glycolytic gene expression) relative to their IL-10-negative counterparts, indicative of an oxidative metabolic program. Further, IL-10-expressing macrophages also expressed high levels of multiple phenotypic and molecular factors associated with M2/alternatively activated macrophage differentiation. Unlike acute LCMV infection, during persistent LCMV infection, these macrophages represent a high proportion of the total macrophage population, suggesting a high likelihood that any interaction between a T cell and macrophage will include these regulatory macrophages and result in the delivery of multiple potent inhibitory signals that decrease antiviral responses in order to limit immunopathology. Interestingly, these data support the concept that emergence of oxidative, regulatory macrophages is a general feature of chronic inflammatory events.

IL-10-expressing DCs similarly exhibit increased mitochondrial membrane potential with no notable change in mitochondrial mass. In vitro studies demonstrated an antagonistic role for IL-10 in the acquisition of the glycolytic program in response to TLR signaling. However, we did not find significant differences in the glycolytic program of IL-10-positive and -negative DCs and B cells in vivo, suggesting that IL-10 signals may not directly suppress glycolytic gene expression in these cells. Alternatively, IL-10 signaling may similarly suppress the glycolytic program in both IL-10-expressing and -nonexpressing DCs and B cells. Importantly, our study identifies a metabolic and gene expression pathway associated with naturally arising iAPCs during a persistent infection.

The tight correlation of iAPCs with viral titers and their similar molecular/transcriptional profiles suggests the existence of specific host sensors of viral replication that, in turn, regulate execution of the iAPC differentiation program. Direct targeting of iAPCs, or such sensors, may provide a potent therapeutic strategy for rapidly and specifically dampening suppressive signals and bolstering T cell responses during viral persistence—without perturbing more global regulatory processes. A further understanding of how iAPCs are generated, and the mechanisms by which they modulate T cell activity, may facilitate development of therapeutic strategies for the restoration of immune responses during persistent infection.

EXPERIMENTAL PROCEDURES

Mice, Virus, and DT Administration

C57BL/6 (wild-type), IL-10-deficient, and CD11c-DTR mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Vert-X, LCMV-GP61-80-specific CD4+ TCR transgenic (SMARTA) and the IL-10/Thy1.1 (10BiT) reporter mice (generously provided by Casey Weaver at the University of Alabama, Birmingham [via Gislaine Martins at Cedars-Sinai Medical Center, Los Angeles, CA]) have been described previously (Madan et al., 2009; Maynard et al., 2007; Oxenius et al., 1998). All mice were housed under specific pathogen-free conditions, and mouse handling conformed to the requirements of the University of California, Los Angeles, Animal Research Committee guidelines. In all experiments mice were infected intravenously (i.v.) via the retro-orbital sinus with 2×10^6 plaque-forming units (PFUs) of LCMV-Arm or LCMV-Cl 13. Virus stocks were prepared and viral titers were quantified as described previously (Brooks et al., 2005). The frequency of productively infected IL-10-expressing splenic DCs (GFP+, CD45+, CD3–, NK1.1–, CD11c+), B cells (GFP+, B220+), and macrophages (GFP+, CD45+, CD3–, NK1.1–, CD11c–, F4/80+) was determined by FACSorting the individual population and performing limiting dilution plaque assays (infectious center assays) on Vero cells (Brooks et al., 2006a). Diphtheria toxin (100 ng) (List Biological Laboratories, Campbell, CA) was administered intraperitoneally on days 5 and 7 after LCMV-Cl 13 infection.

Bone Marrow Chimera Experiments

C57BL6 or IL-10-deficient recipient mice were lethally irradiated with 950 rads and on the same day received 20 million total bone marrow cells intravenously isolated from the femurs and tibia of either IL-10-deficient or WT donor mice. Recipient mice were treated with antibiotics (Sulfamethoxazole and Trimethoprim in the drinking water) for 3 weeks to prevent infection and allow for hematopoietic reconstitution. Eight weeks following bone marrow transfer, mice were bled to confirm consistent reconstitution and then infected with LCMV Cl13.

Flow Cytometry

Analysis of IL-10-expressing (GFP+) and nonexpressing (GFP–) cells was performed by staining directly ex vivo for surface expression of CD45-Pacific Orange or Pacific Blue; CD11c-Pacific Blue or PE; Thy1.1-FITC or PE; Thy1.2-PerCP, NK1.1-PerCPCy5, B220-APCCy7, CD11bPeCy7, F4/80-PE, or APC; PDL1-PE or biotin followed by streptavidin-APC, PDL2-PE, MHC Class II-PE, MHC Class I-PE, CD80-PE, CD86-PE, CD4-Pacific Blue, CD8-Pacific Blue. Mitochondrial membrane potential was accessed by staining with MitoTracker FM Red (Invitrogen) according to the manufacturer's instructions. MHC tetramers were obtained from the National Institutes of Health (NIH). Flow cytometric analysis was performed using the Digital LSR II (Becton Dickinson) in the UCLA Jonsson Comprehensive Cancer Center (JCCC)/Center for AIDS Research Flow Cytometry Core Facility (NIH grant CA-16042).

Purification of APC Populations

IL-10-producing and nonproducing DCs and macrophages were sorted from spleen following B cell depletion (CD19 MACS beads, Miltenyi) as follows: IL-10-expressing DCs (GFP+, CD45+, Thy1.2–, NK1.1–, CD11c+ bright, CD11b+), non-IL-10-producing DCs (GFP–, CD45+, Thy1.2–, NK1.1–, CD11c+ bright, CD11b+), IL-10-expressing macrophages (GFP+, CD45+, Thy1.2–, NK1.1–, F4/80+), and non-IL-10-producing macrophages (GFP–, CD45+, Thy1.2–, NK1.1–, F4/80+). The depleted B cell (CD19+) fraction was subsequently sorted for IL-10-producing (GFP+, CD45+, Thy1.2–, NK1.1–, B220+) and nonproducing (GFP–, CD45+, Thy1.2–, NK1.1–, B220+) populations. Cells were sorted using a FACS Aria or Vantage fluorescence-activated cell sorter (Becton Dickinson). Postsort purity was >98%.

Quantitative RT-PCR

RNA purified from whole splenocytes or sorted APC populations was isolated with the RNeasy Extraction Kit (QIAGEN). RNA was normalized for input and amplified directly using the One-Step RT-PCR Kit (QIAGEN). *IDO*, *TGF-β*, *IL-10*, *IL-12*, *Blimp1*, *IRF4*, *IRF3*, *BCL6*, and *HPRT* were amplified using Applied Biosystems Assays-on-Demand TaqMan premade expression assays. *IDO*, *TGF-β*, and *IL-12* expression were normalized to *HPRT*. Metabolic gene expression was determined from cDNA with the exception that SYBR Green (Roche) real-time quantitative PCR assays were performed using the Roche Light cycler 480 II (Roche). Genes are normalized to ribosomal housekeeping gene Rip140. The primer sequences for the metabolic genes are available upon request.

In Vitro T Cell Stimulation

Antigen-specific naive CD4+ T cells or CD8+ T cells were isolated from the spleens of SMARTA or P14 mice, respectively, and purified by negative

selection (StemCell Technologies). T cells were then labeled with 2.5 uM CFSE (Invitrogen) and cultured for 3 days with sorted populations of IL-10-producing (GFP+) or non-IL-10-producing (GFP−) APCs at a ratio of 2:1 (40,000 T cells/20,000 APC). In some situations, 10 μg/ml anti-IL10R antibody (clone 1B1.3A; BioXcell) or anti-PDL1 antibody (clone 1F.9G2, BioXcell), or 200 μM 1-methyl tryptophan (1-MT; Sigma-Aldrich) was added to the cultures. The frequency of SMARTA or P14 cells that proliferated during culture was determined as described (Gett and Hodgkin, 2000). Briefly, the number of cells in each division peak was divided by 2^i (where i equals the number of divisions). The total number of SMARTA cells in each peak was then summed, and this number was divided by the total number of SMARTA cells in the culture. This number was then expressed as the percent of cells that had proliferated. To obtain the stimulation index, the total number of T cells that proliferated in the cultures of GFP+ culture was divided by the number of T cells that proliferated in the GFP− culture, multiplied by 100.

ELISA

Plasma IL-10 levels were determined using the Quantikine IL-10 Elisa kit (R&D Systems, Minneapolis, MN). Optical density values were read using a Synergy 2 plate reader (BioTek, Winooski, VT) at 450 nm.

Statistical Analysis

Student's t tests (two-tailed, unpaired) were performed using the GraphPad Prism 5 software (GraphPad Software Inc.).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article at doi:10.1016/j.chom.2012.03.009.

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